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Virus Isolation and Propagation from H3N2 Influenza Infected Human Clinical Samples Under Distinct Sample Storage Conditions

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Abstract: Isolation and propagation of influenza virus from Influenza Like Illness (ILI) clinical sample is essential for the surveillance of circulating virus, such as antigenic and genetic analyses, antiviral sensitivity surveillance, as well as annual influenza vaccine selection. Madin-Darby canine kidney (MDCK) cell is conventionally used for virus isolation in public health laboratories. Throat swap samples of Influenza like Illness (ILI) were collected from two sentinel hospitals and screened seasonal influenza by real-time reverse transcription polymerase chain reaction (RT–PCR). H3N2 positive samples were performed virus isolation in MDCK cells. Samples were stored under different conditions before inoculation, 1-2 days at 2-8°C, 4-5 day or 8-9 days at 2-8°C, and no less than two months at -80°C. The results showed that long term (>2 month) -80°C storage of clinical samples (15.12%) had significantly lower virus isolation rate compare to short term (1-2 days and 4-9 days) under 2-8°C storage (88.37% for 1-2 days and 52.33% for 4-9 days). For those samples stored at 4°C, the shorter of the storage time, the better of sample quality and virus activity could be obtained, resulting in higher isolation rate. This study provides evidence for influenza surveillance and sample quality control.

Keywords: Influenza, Virus Isolation, Sample Quality, Sample Storage Condition

1. Introduction

Influenza virus antigenic changes of glycoproteins can result in circulating of different variants of influenza virus. The continuous evolution of influenza viruses is monitored by the WHO Global Influenza Surveillance and Response System (GISRS) [1-4]. One of the main roles of this network is to monitor the evolution of influenza viruses and select candidate vaccine viruses for vaccine production.

Isolation and propagation of influenza virus from infected human host sample is essential for the yearly surveillance of circulating virus and for further studies, such as antigenic and antiviral sensitivity analyses, as well as annual influenza vaccine selection. Influenza viruses can be isolated using embryonated chicken eggs or several different cell-lines, such as rhesus monkey kidney (RhMK), the African green monkey kidney (AGMK/Vero), Madin-Darby canine kidney (MDCK), mink lung epithelial (Mv1Lu), rhesus monkey kidney (LLC MK2), and buffalo green monkey kidney (BGMK) cell-lines [5]. A number of seasonal influenza viruses replicate poorly in eggs [6, 7], especially H3N2 viruses. Therefore, multiple mammalian cells were used to isolate influenza viruses from clinical samples. Among these cell-lines, the MDCK cells, which is easy to handle, sensitive and reliable, have been used extensively in surveillance and research of influenza viruses [8-11].

Influenza A and B viruses bind to sialyloligosaccharides on host cell surface glycolipids or glycoproteins via the hemagglutinin (HA) protein, a surface spike protein on virions. Human influenza viruses preferentially bind to sialyloligosaccharides containing terminal *N*-acetyl sialic acid linked to galactose by anα-2,6-linkage (NeuAc_2,6Gal), while avian influenza viruses mainly bind to those containing anα-2,3-linkage (NeuAc_2,3Gal) [12, 13]. Both human and avian influenza viruses can be isolated from and propagated in MDCK cells with high viral titers [14-16], which may be attributed to the fact that both α-2,6- and α-2,3-linked sialic acid receptors are expressed on the surfaces of MDCK cells [13, 17]. Due to the distinction of receptor binding sites in different propagation substrate, influenza viruses can acquire HA and NA mutations when passaged in tissue culture or eggs; however, we do not fully understand whether these adaptive mutations influence its antigenicity.

Sample quality, which could influence virus isolate rate, is an important guarantee for surveillance sensitivity and accuracy [18]. There are many subjective factors affecting the quality of samples, including the quality of sample collection, preservation and transportation conditions, etc. [19, 20]. In this study, the virus isolation rate was detected to evaluate virus activity and stability in clinical samples, so as to provide scientific evidence for improvement of virus isolation rate and influenza surveillance quality.

2. Method

2.1. Cells

MDCK cells were passaged in Dulcecco's modified Eagle's medium (gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 10 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin. MDCK cells were maintained at 37°C and 5% CO_2.

2.2. Sample Collection and Study Design

Two sentinel hospitals in Beijing were selected in this study. Throat swabs of ILI patients were collected within three days of onset by clinical doctors in the hospitals. Commercial swabs and sample storage medium (Youkang, MT0301) were used in the two hospitals. Samples were stored and transported to prefectural CDCs under the condition of 2-8°C within 48hs after collection. Then the samples were aliquoted into 4 vials, one was for ILI surveillance detection and another three were transported to Chinese National Influenza Center (CNIC) within 4hs for this study. Sample transportation and storage temperature was whole-process monitored by Testo 184 data logger. In CNIC, samples were preliminarily screened by real-time RT PCR using seasonal influenza detection primers and probes right after CNIC received the samples [18]. Positive samples were then selected for virus isolation. All the nucleic acid positive samples were inoculated in MDCK cells at first time point (TP1, day 1-2), second time point (TP2, day 4-5 or day 8-9), and third time point (TP3, two months later) after sample collection. All samples tested in TP1 and TP2 were stored at 2-8°C after sample collection, while TP3 samples were stored at -80°C after sample reception. (Figure 1).

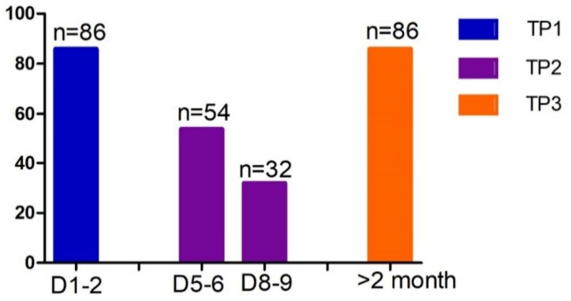

Figure 1. *The number of clinical samples tested at different time points.*

2.3. Nucleic Acid Detection

Nucleic acid was detected as previously described [18]. Nucleic acid was extracted from 200 μl samples using MagMAX™ CORE Nucleic Acid Purification Kit on MagMAX_Core_Flex automatic machine. RNA was eluted in 50μl elution buffer. All samples were preliminarily screened by real-time RT-PCR (SuperScrip^{TM} III one-step RT-PCR System) using published primers and probes for the influenza A matrix (M) gene and influenza B and H1 Heamagglutination (HA) genes [21].

2.4. Virus Isolation

MDCK cells were washed twice with Ca^{2+}/Mg^{2+} free phosphate-buffered saline (PBS) before incubation with 500 μl samples at 35°C and 5% CO_2 for 1 h. After inoculation, 5 ml medium (without FBS) supplemented with 4 μg/ml trypsin and 10% bovine serum albumin (BSA) was added. The cells were incubated at 35°C and 5% CO_2 for 4 days. The supernatant was collected and the presence of virus was assessed by hemagglutination using 1% turkey or guinea pig red blood cells (RBC). Negative samples in the first isolation passage were passaged one more time in MDCK cells. The negative samples in both of the passages were recorded as negative.

2.5. HA gene Sequencing and Sequence Analysis

Full genome sequencing of HA gene were performed by Shanghai Bo Jie company. A phylogenetic tree of HA gene was constructed by neighbor joining method with 500 bootstrap replicates using MEGA7.0 software.

2.6. Statistic Analysis

Isolation rates were analyzed by Cochran's Q test and Person's chi-square test using SPSS software. A P value of < 0.05 was used to indicate a significant difference.

3. Results

A total of 201 clinical samples were collected between December 2019 to January 2020 from two hospitals in Beijing, China. Samples were screened by Real time RT-PCR. Eighty-six samples were flu A positive, where seven samples

were H1N1 positive and seventy-nine samples were H3N2 positive. There was no flu B positive sample. Figure 2-A shows positive isolates number at different time points. A total of seventy-seven strains were isolated from the eighty-six fluA positive samples. The overall isolation rate was 89.53%. The isolation rates in each group were significantly different that were 88.37% (76 out of 86) in TP1, 52.33% (45 out of 86) in TP2 and 15.12% (13 out of 86) in TP3 (χ^2=86.290, p=0.000). eight samples were obtained positive isolates in all the three groups, however, nine samples were not obtained positive isolates in any of the three groups. Thirty-six samples were isolated in TP1 and TP2, but not TP3 and twenty-seven samples were isolated in TP1, but not TP2 and TP3. Five of the samples obtained positive isolates in TP1 and TP3, but not TP2. Only one sample got positive isolates in TP2 but not in TP1 and TP3.

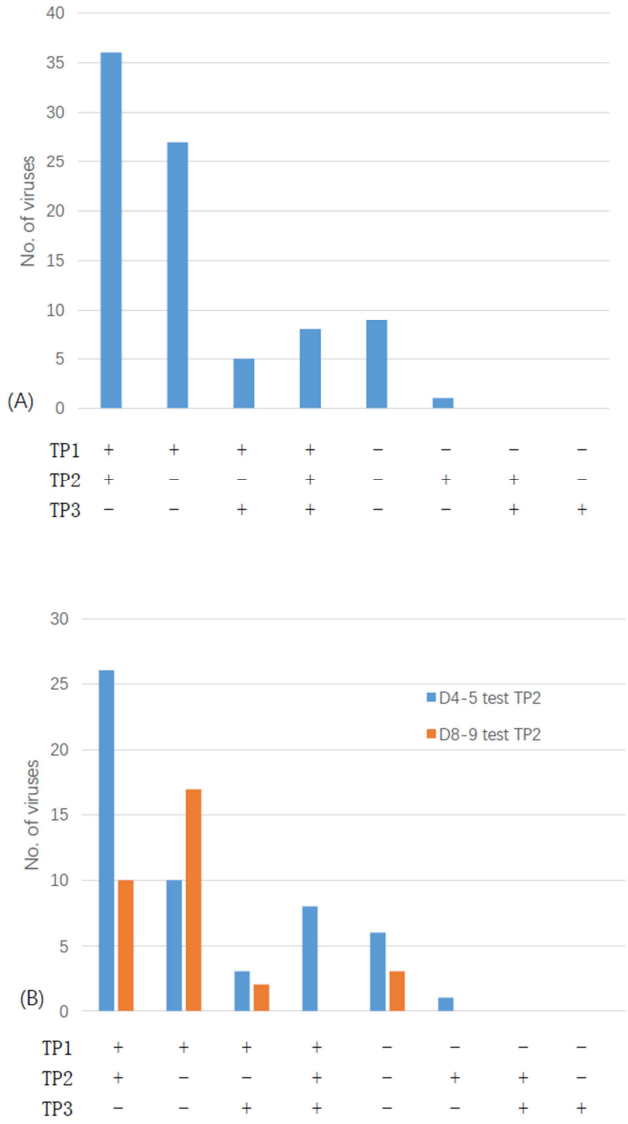

Figure 2. *Virus isolation in different groups.*

(A) Number of viruses isolated in different status. (B) Comparison of virus isolation between Day 4-5 and Day 8-9. Blue bars represent the number of virus isolates derived from those samples isolated at day 4-5 in TP2. Orange bars represent the number of virus isolates derived from those samples isolated at day 8-9 in TP2.

Due to the limitation of sample volume, samples were not able to test in more than three groups. TP2 samples were tested at two different time points that fifty-four samples were tested at day 4-5 and thirty-two samples were tested at day 8-9 in TP2. Figure 2-B shows isolation number at different circumstances. Overall, the positive isolation rate in TP2 samples tested at day 8-9 was significantly decreased compared to the TP2 samples tested at day 4-5 (χ^2=9.074, p=0.003). In the fifty-four samples, which were isolated at day 4-5 in TP2, 48.15% (n=26) samples got positive isolates in TP1 and TP2, but negative isolates in TP3 and 18.52% (n=10) samples got positive isolates in TP1, but negative in TP2 and TP3. However, the ratio is opposite in those thirty-two samples that were isolated at day 8-9 in TP2. 31.25% samples (n=10) were got positive isolates in TP1 and TP2, but negative isolates in TP3 and 53.13% (n=17) samples got positive isolates in TP1, but negative in TP2 and TP3. Eight samples, which were isolated at day 4-5 for TP2 had positive isolates in all three groups. While for those samples isolated at day 8-9 for TP2, none positive isolates in all three groups was identified.

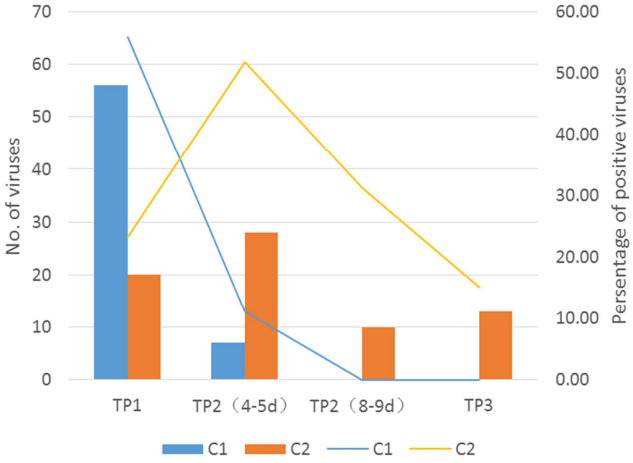

Figure 3. *Comparison of positive isolation between different passages.*

Blue bars represent the number of viruses isolated in the first passage. Orange bars represent the number of viruses isolated in the second passage. Blue line represents percentage of positive viruses isolated in the first passage. Orange line represents percentage of positive viruses isolated in the second passage.

All the samples were isolated in MDCK cells for two passages. We compared the isolation results from the two passages in different groups (Figure 3). The isolation rate in the first passage was 65.12% in TP1. While it was dramatically decreased to 12.96% in TP2. No virus was isolated from the first passage in TP3.

A total of 67 HA genes of isolated viruses were sequenced. The phylogenetic tree showed that 41 HA genes belong to 3C.2a1b+T135K-B with same amino acid change at S137F, A138S and F193S, and 25 HA genes belong to 3C.2a1b+T135K-A with same amino acid change at A138S, G186D, D190N, F193S, S198P (Figure 4). This is consistent with the surveillance data in China and globally that 3C.2a1b+T135K-A and 3C.2a1b+T135K-B group viruses were co-circulating and predominate for H3N2 in 2019-2020 influenza season.

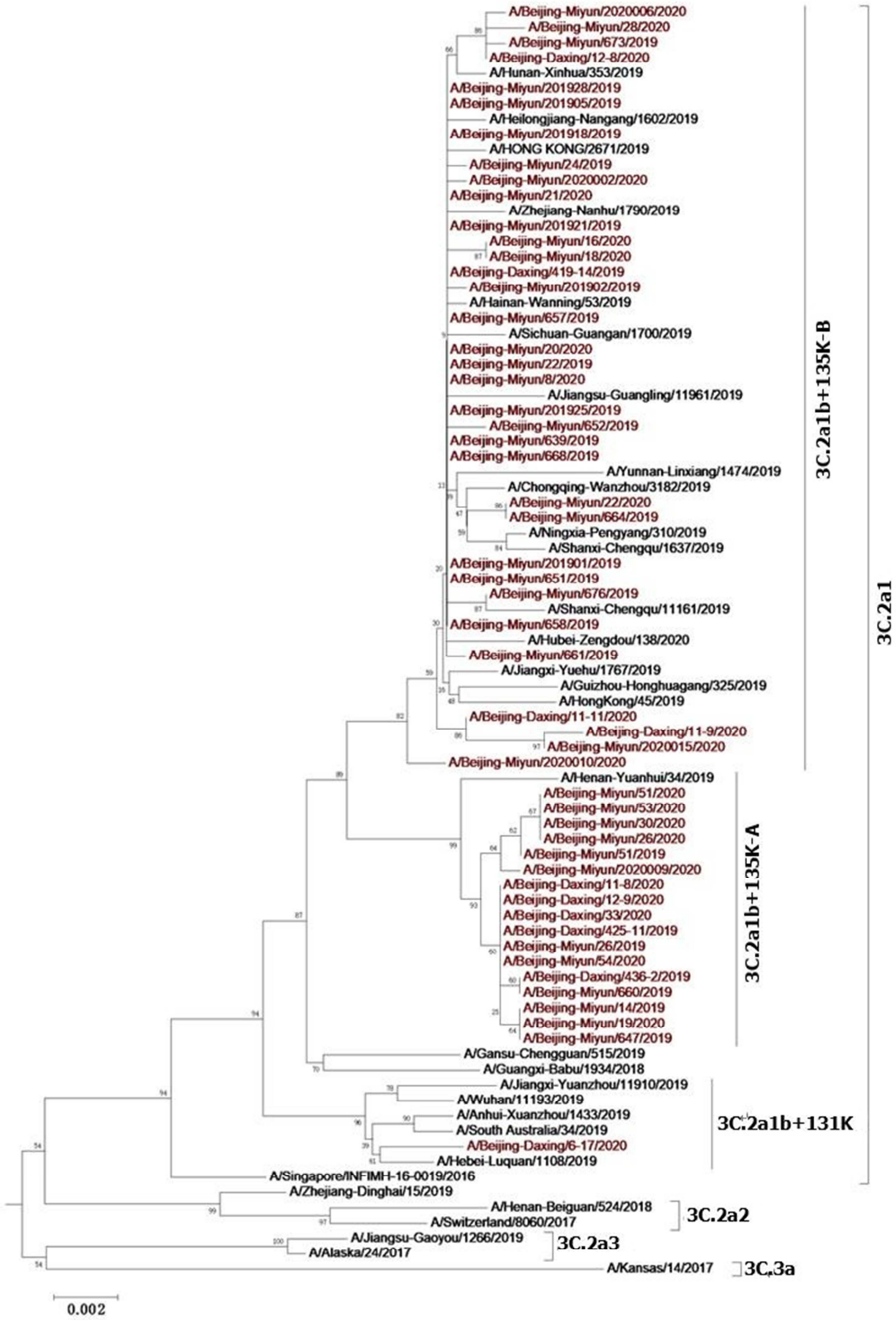

Figure 4. *Phylogenetic tree of H3N2 viruses on the basis of HA sequences.*

The phylogenetic tree of HA gene was constructed by neighbor joining method with 500 bootstrap replicates using MEGA7.0 software. Red viruses were isolated in this study. Black viruses were randomly selected and download from GISAID. Genetic groups were labeled. Scale bar indicates nucleotide substitutions per site.

4. Discussion

Virus isolation and propagation from influenza infected clinical samples is essential for routine influenza surveillance, such as antigenic and antiviral sensitivity analyses, as well as annual influenza vaccine selection. Embryonated eggs and MDCK cells are routinely used for virus propagation by WHO Collaborating Center for Reference and Research on Influenza [22]. Host adaptive mutation could be occurred when virus is passaged in tissue culture or embryonated eggs. Some of the adaptive mutations on HA and NA genes may influence antigenic characteristic [23]. This is due to mismatch of influenza viruses with receptors on cell surface. For H3N2 viruses, egg adaptive mutations on HA gene was continually observed in virus isolates, resulting in antigenic change in conventional hemoglutination inhibition (HI) assay. And also, some influenza viruses, especially H3N2 viruses, replicate poorly in eggs [6, 7]. Therefore, many laboratories replaced it with mammalian cells for the primary isolation of influenza viruses from clinical samples [24]. MDCK cell, which is easier to handle and access and has higher isolation rate, is more commonly used in public health laboratories. Virus isolation efficiency of clinical influenza sample in different MDCK cells were evaluated previously [16, 25-30]. Distinct from embryonated chicken eggs, MDCK cells present both NeuAc_2,6Gal and NeuAc_2,3Gal receptors [17]. In order to simulate epithelial cells in human airway, a number of modified MDCK Cells with enhanced expression of NeuAc_2,6Gal-Linked Sialic Acid are used for influenza virus propagation [26, 31-33]. These 2,6-Linked Sialic Acid over expressed MDCK cells also suitable for assessing the NA inhibitor susceptibility of human influenza viruses [22].

In addition, sample isolation rate could be significantly influenced by sample quality. Distinct from nucleic acid detection, virus isolation requires live influenza virus but not only the existence of nucleic acid fragment. Therefore, virus isolation rate is normally much lower than nucleic acid detection. For different subtypes or different genetic groups of influenza viruses, the virus isolation rate could vary widely. Sample quality could be influenced by the quality of sample collection, preservation and transportation conditions, etc.

In this study, sample isolation rate at different storage conditions after sample collection was compared. For each sample, three storage conditions were selected that 2-8°C for 1-2 days (TP1), 2-8°C for 4-5 days or 8-9 days (TP2), and -70°C for no less than 2 months (TP3). Two passages were conducted for each test. The results showed that virus isolation rate was dramatically influenced by storage condition. Store at -70°C for a couple of months could significantly impact virus activity and decrease isolation rate.

5. Conclusion

Sample storage condition, which is essential to sample quality, could significantly influence influenza virus isolate rate. The virus isolation rate of H3N2 virus from clinical samples stored at 2-8°C for short time period is higher than the samples stored at -70°C for long time period. Sample storage condition at 2-8°C for a short period should be preferred.

Acknowledgements

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